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SUGAR TRANSPORT IN REVERSIBLY HEMOLYZED AVIAN ERYTHROCYTES

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SUMMARY

The technique of reversible hemolysis represents one approach which may be used to study transport regulation in nucleated red cells. After 1 h of incubation at 37 °C, 88 % of the ghosts regained their permeability barrier to L-glucose. In these ghosts, the carrier-mediated rate of entry of 3-*O*-methylglucose was more than 10-fold greater than the rate in intact cells. Glyceraldehyde-3-phosphate dehydrogenase prevented ghosts from resealing when it was present at the time of hemolysis. Albumin, lactic dehydrogenase and peroxidase did not have this effect. Sugar transport rate could not be tested in the unsealed ghosts. Two possible mechanisms for the effect of hypotonic hemolysis on sugar transport rate were discussed: (1) altered membrane organization and (2) loss of intracellular compounds which bind to the membrane and inhibit transport in intact cells.

INTRODUCTION

The mechanism of regulation of carrier-mediated sugar transport in eukaryotic cells has been under investigation for some time [1–5]. Many factors have been identified which lead to a change in transport rate [6], and levels of several intracellular metabolites have been shown to change with transport acceleration [4]. In spite of continued work, the identity of a regulator or regulating mechanism is not known.

The technique of reversible hemolysis represents one approach which may be used to study transport regulation in nucleated red cells. A previous report [3] suggested that sugar efflux was stimulated in osmotically shocked avian erythrocytes, perhaps due to loss of an intracellular transport regulator. In the present studies, sugar transport in reversibly hemolyzed avian erythrocytes was investigated under conditions where the population of cells was more uniform and where sugar entry could be determined and corrected for the portion of cells which did not reseal after hemolysis. In addition, glyceraldehyde-3-phosphate dehydrogenase, an enzyme known to bind to human red cell membranes [7, 8], was tested as a potential regulator of transport. The effect of glyceraldehyde-3-phosphate dehydrogenase on hemolysis and resealing of erythrocytes was also determined.

EXPERIMENTAL PROCEDURE

Blood was collected from the neck veins of white domestic geese, into polycarbonate bottles containing heparin (2 mg/100 ml blood). The cells were washed three times in cold buffer, resuspended and stored overnight at 4 °C. Cells were washed again immediately before use. Leukocytes were removed during the washing procedure. Buffer composition included 137 mM NaCl, 5.9 mM KCl, 1.3 mM $CaCl_2$, 2.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM imidazole, 7.6 mM glycylglycine, and after the first wash, 0.2 % bovine serum albumin.

Reversible hemolysis

20 g of packed red cells were added quickly to 400 ml of rapidly stirring hemolyzing solution, pH 6.0, at 1 °C. Composition of the hemolyzing solution was 4.99 mM imidazole glycylglycine, 2.0 mM $MgSO_4$, 1.0 mM $CaCl_2$ and NaCl, to bring the final osmolarity to 54 mosM/l. The cell suspension was continuously stirred for 3.0 min at 1 °C, and then the osmolarity rapidly restored to 300 mosM/l by addition of 20 ml of a solution containing 2.5 M KCl, 0.24 M $MgCl_2$ and 0.1 M $CaCl_2$. The pH was adjusted to 7.4. Restored ghosts were healed for various periods of time up to 90 min by incubation in the hemolysate at 37 °C in a New Brunswick Rollertherm incubator. After the healing period, ghosts were collected by centrifugation and washed three times with cold buffer.

When the hemolysis was done in the presence of protein, the stock protein, in 3 M ammonium sulfate, was dialyzed overnight in the cold, against several changes of hemolyzing solution, 40–50 mosM/l, pH 7.4, to remove the salt. The dialysate was added to fresh hemolyzing solution, to a final concentration of 0.01–1.0 mg/ml, and the osmolarity adjusted to 54 mosM/l, pH 6.0.

3-O-methylglucose entry

Ghosts were resuspended in buffer, to an approximate hematocrit of 30 %. Aliquots of the suspension were taken for cell counts and volume measurements on a Coulter Counter Z_{BI} (70 μM aperture tube) with Channelyzer and Plotter attachments. Other aliquots of the suspension were taken for measurement of L-[^{14}C]inulin ($M_r \sim 5000$) and [^{14}C]dextran ($M_r \sim 75\,000$) spaces. The aliquots were thoroughly mixed in the cold with the labeled substances and samples were withdrawn for ^{14}C determination in the extracellular medium and in the water of the whole suspension. Samples of the whole suspension were lysed, and deproteinized with perchloric acid before counting. Spaces (volumes of distribution) were defined and calculated as previously described [3].

The remainder of the suspension was incubated with 5–10 μM 3-*O*-[^{14}C]-methylglucose in an open flask at 37 °C in a shaking water bath. After various periods of time from 0 to 20 min, duplicate samples were withdrawn and transport stopped by transferring the suspension to ice cold tubes. The suspension was allowed to cool, and the ghosts collected by centrifugation and washed three times. The ghosts were lysed with water and deproteinized with perchloric acid. Aliquots of the extract were counted to determine 3-*O*-methylglucose entry. Samples of the whole suspension were also lysed, deproteinized and counted to determine 3-*O*-methylglucose concentration in the total water of the suspension. Total water of the suspension was determined by drying aliquots at 90 °C for 24 h.

Equilibration of sugar in the intracellular and extracellular water was calculated as previously described [4]. Half-times ($t\frac{1}{2}$) of 3-*O*-methylglucose entry were derived from a plot of log (1-fraction equilibrated) as a function of time. Rates of 3-*O*-methylglucose entry were determined from the following equation [9]:

$$\frac{S_0}{V_s} = \frac{\left(\frac{1}{W_0} + \frac{1}{W_i}\right)(t\,\tfrac{1}{2})\,(\text{number of competent ghosts} \cdot 10^{-9}/\text{ml})}{0.693} \qquad (1)$$

S_0 was the concentration (μM) of 3-*O*-methylglucose in the extracellular fluid, and V_s was the rate of sugar entry (pmol/10^9 ghosts · min^{-1}). Extracellular water volume, W_0, was taken as the L-glucose space (ml/ml suspension). Intracellular water volume, W_i, was defined as the difference between the total water of the suspension and the L-glucose space. The total number of ghosts was $1\text{–}2 \cdot 10^9$ per ml suspension in various experiments. The number of ghosts that resealed and did not allow entry of L-glucose were termed competent ghosts. Total intracellular water was taken as total water per ml of suspension minus the dextran space (ml/ml suspension). L-glucose accessible intracellular water, the L-glucose space minus the dextran space, represented the volume of the population of leaky ghosts. The number of competent ghosts was then calculated from:

$$\left(1 - \frac{\text{L-Glucose space–dextran space}}{\text{total waterd–extran space}}\right) \times \text{number of ghosts/ml suspension.}$$

These space measurements assumed that entry of L-glucose occurred by diffusion but not by the 3-*O*-methylglucose or D-glucose carrier, and that dextran did not penetrate the membrane of healed ghosts (see Discussion).

The activity constant of the carrier, F_s, as defined previously by Regen and Morgan [9] was calculated as:

$$F_s \cong \frac{V_s}{S_0}$$

This constant represents the overall rate constants for combination of sugar and carrier on one side of the membrane, leading to appearance of sugar and carrier on the other side. The equation for determination of F_s applied in these experiments since only one sugar was present at tracer concentrations.

Glyceraldehyde-3-phosphate dehydrogenase activity

Glyceraldehyde-3-phosphate dehydrogenase activity was determined in aliquots of hemolyzing fluid, and in ghost and intact cell hemolysates by enzymatic assay [10]. The buffer, pH 8.0, contained 32 mM sodium pyrophosphate, 5.5 mM sodium arsenate, 0.6 mM nicotinamide adenine dinucleotide and 3 mM cysteine. Fructose diphosphate (1 mM) and aldolase (5 mg/ml) were added to the cuvette to generate the substrate. Inactive glyceraldehyde-3-phosphate dehydrogenase was prepared by incubating the active enzyme in 0.1 M Tris, pH 7.5–8 for 10 min with iodoacetic acid [11]. The ratio of iodoacetic acid to glyceraldehyde-3-phosphate dehydrogenase was 100 : 1 (mol : mol). After 10 min, dithiothreitol was added in a concentration equimolar to iodoacetic acid. The mixture was dialyzed overnight to

remove excess inactivator. Enzyme activity is expressed as U/ml or U/10^9 ghosts, where U represents the conversion of 1 μmol of substrate/min.

Cell counts and sizing

Cells or ghosts were diluted with buffer which had been passed through a 0.22 μm Millipore filter. The dilution was such that the total number of particles was 2000–5000/0.1 ml to minimize coincidence. The Coulter Counter base channel threshold was 10 and the window width was 100. The matching switch was set to 20 k. Ghosts were sized with the edit circuit off, and usually with the aperture current at 0.125–0.25 mA.

Reagents

All inorganic chemicals were reagent grade. Organic reagents and enzymes were obtained from Sigma or Calbiochem. Glyceraldehyde-3-phosphate dehydrogenase was A-grade obtained from rabbit muscle. Radioisotopes were obtained from New England Nuclear.

RESULTS

Ghost properties

Avian erythrocytes have not been as extensively investigated as human red cells in regard to the methods of producing ghosts and the time required for resealing the membrane after hemolysis. The presence of Ca^{2+} and Mg^{2+} at higher concentrations than those used by others [12, 13] in the hemolyzing and restoring solutions facilitated resuspension of the ghosts during washing. Ghost populations were more homogeneous in appearance and more uniform in shape and size when inspected by phase contrast microscopy, if the temperature and pH at the time of hemolysis were 1 °C and pH 6.0. These conditions were the same as those reported for human erythrocytes [13, 14]. Ghost cells had a dry weight of 0.07–0.10 g/ml suspension, compared to a value of 0.360–0.40 for intact cells.

If ghost suspensions were not healed at 37 °C, the volume of distribution of L-glucose approximated the total volume of water of the whole suspension (Fig. 1). After 30, 60 and 90 min of healing, L-glucose, inulin and dextran spaces decreased, with the greatest change between 0 and 30 min. Ghosts which were held at 0 °C for 60 min had L-glucose and dextran spaces identical to those of newly restored, unhealed ghosts. There were no significant differences between dextran and inulin spaces in the ghost suspensions. The presence of albumin (1 mg/ml) in the hemolyzing medium did not affect the rate of healing or the initial permeability of the restored ghosts. Based on these observations, a healing time of 60 min was chosen for subsequent experiments. At this time, 88.1 $\pm$ 3.7 % (n = 10) of the ghosts were competent.

Comparison of mean cell volume in ghosts and intact cells indicated that the ghost was approximately 30 % larger. These calculations were made by two methods: (1) 1-dextran space (ml/ml suspension)/number of cells per ml suspension; (2) from Coulter Counter measurements using paper mulberry pollen (12–14 μm diameter), latex spheres (9.6 μm diameter) and human red cells (90 μm^3 mean cell volume) as standards. Mean cell volumes for intact cells calculated from the two methods were

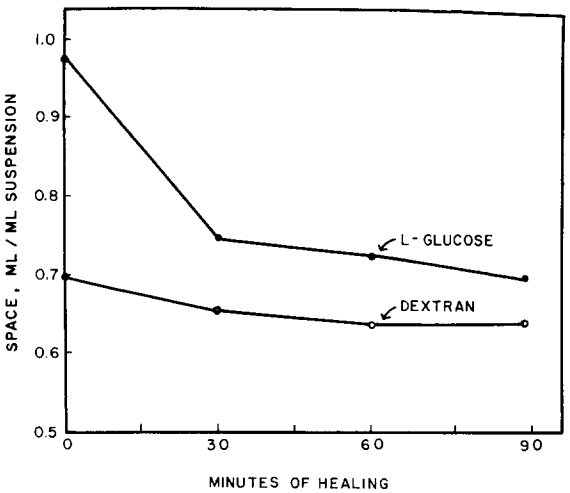

Fig. 1. Effect of time allowed for resealing on L-glucose and dextran spaces of ghost suspensions. Cells were reversibly hemolyzed, healed for various times at 37 °C, and the L-glucose (●) and dextran (○) spaces determined as described in Experimental Procedures. The number of ghosts/ml suspension was $1.75 \cdot 10^9$. The experiment has been repeated several times with different batches of hemolyzed cells, and identical time courses were obtained.

146 ± 7 and 145 ± 4 μm^3 respectively. Comparative volumes for ghosts were 187 ± 10 and 189 ± 13 μm^3 respectively. Apparent particle volumes read from the Coulter Counter varied with the standards used. Human red cells are known to have a mean cell volume of 90 μm^3. When latex particles were used as a reference, the mean cell volume of these cells was underestimated by 40 %. Use of pollen as a reference led to a 27 % overestimation of mean cell volume. Intact cell and ghost mean cell volumes were corrected for these discrepancies. The mean cell volume of intact cells was very

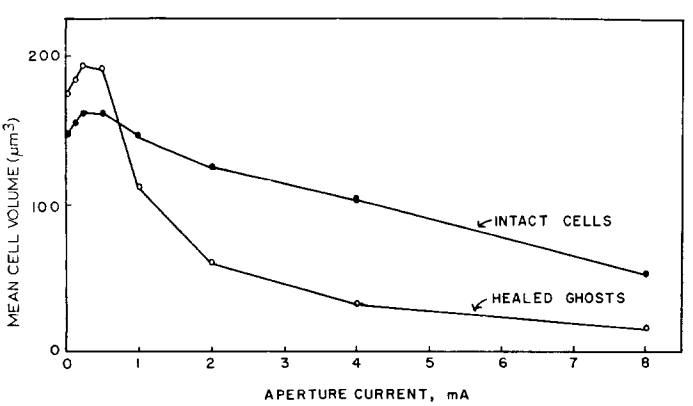

Fig. 2. Apparent mean cell volumes of intact cells and ghost suspensions measured at increasing detector currents. Intact cells (●) or ghosts (○) which had been healed for 60 min at 37 °C were washed, resuspended and counted on the Coulter Counter. Volume measurements were taken at various aperture current settings, with 12–14 μm diameter pollen and human red cells as standards. This experiment has been repeated several times with the same results.

similar to the value of 142 μm^3 reported for duck erythrocytes [15].

The mean cell volume derived from the Coulter Counter also depended upon the aperture current chosen to count and size the cells. (Fig. 2). The apparent mean cell volume increased slightly when the current increased from 0.063 to 0.25 mA. Above this value apparent mean cell volume decreased with increasing current. The decrease in mean cell volume was significantly greater than in intact cells. Cell number did not vary with aperture current. Based on these observations, a current of 0.125–0.25 mA was used for routine comparisons of mean cell volume and cell numbers.

Transfer of 3-O-methylglucose

Entry rate of tracer concentrations of 3-*O*-methylglucose in ghosts was 10-fold greater than that in intact cells (Table I). There was no difference in transport rate whether the ghosts were prepared in the presence or absence of albumin. The activity constant was also 10-fold greater in the ghosts. Phloretin, a competitive inhibitor of 3-*O*-methylglucose transport, suppressed the entry rate 5–6 fold in ghosts. The inhibitable portion of sugar entry was a greater fraction of total sugar entry than in intact cells. This suggested that most of the increased rate of 3-*O*-methylglucose entry involved stimulation of carrier-mediated transport.

TABLE I

3-*O*-METHYLGLUCOSE ENTRY IN INTACT CELLS AND GHOSTS

Intact cells and ghosts were prepared, incubated with tracer 3-*O*-methylglucose concentrations, and the entry rate determined as described in Experimental Procedure. Symbols have the following meaning as defined in Experimental Procedures: S_0 = extracellular 3-*O*-methylglucose concentration; N = number of experiments and ghosts represent competent particles. V_s = 3-*O*-methylglucose entry rate; F_s = activity constant. The data are expressed as the mean ± S.E.M. $a = P < 0.001$ compared to intact cells.

Cell type	S_0 (μM)	N	Phloretin (0.5 mM)	V_s (pmol/10^9 ghosts · min^{-1})	F_s (μl/10^9 ghosts · min)
Intact cells	6.44 ± 1.07	7	0	2.57 ± 0.37	0.42 ± 0.04
		2	+	1.91 ± 0.19	0.32 ± 0.03
Ghosts	7.00 ± 1.73	6	0	32.3 ± 5.0[a]	5.09 ± 0.77[a]
		2	+	5.16 ± 1.06	0.91 ± 0.22
Ghosts with albumin	5.07 ± 0.84	5	0	35.0 ± 5.5[a]	6.67 ± 0.68[a]
		3	+	4.56 ± 1.23	1.04 ± 0.04

Influence of glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase is an important regulatory enzyme in glycolysis and is also known to bind reversibly to human red cell membranes [7, 8]. It therefore might be a regulator of sugar transport. Glyceraldehyde-3-phosphate dehydrogenase activity in intact avian erythrocytes was 0.787 ± 0.088 (n = 6) U/10^9 cells when assayed in the presence of 1.0 % Triton. A similar value has been found for glyceraldehyde-3-phosphate dehydrogenase activity in human erythrocytes [16]. Glyceraldehyde-3-phosphate dehydrogenase activity could not be detected in ghosts prepared in the absence of added enzyme. When the enzyme was included in the hemolyzing fluid, glyceraldehyde-3-phosphate dehydrogenase activity was incor-

porated into the ghosts, a result which has also been reported for human red cells hemolyzed in the presence of enzymes [17]. Hemolyzing fluid containing glyceraldehyde-3-phosphate dehydrogenase activities of approximately 1 and 3 U/ml (0.25 and 0.5 mg protein/ml) yielded ghosts which, after healing and subsequent washing, had activities of approximately 0.2 and 0.4 U/10^9 ghosts.

Cells hemolyzed in the presence of glyceraldehyde-3-phosphate dehydrogenase were not able to reseal completely when the enzyme concentration was greater than 0.25 mg/ml of hemolyzing fluid (Fig. 3). At an enzyme concentration of 1 mg/ml, virtually all of the water of the ghost suspension was available to L-glucose. Both the L-glucose and dextran spaces increased with an increase in enzyme concentration, although there was no increase in total water in the suspension and no loss of total ghost particles. Cells hemolyzed in the presence of bovine serum albumin, lactic dehydrogenase or peroxidase (1 mg/ml) showed no inhibition of resealing. The effect of glyceraldehyde-3-phosphate dehydrogenase and albumin on healing of ghosts is shown in Fig. 4, for suspensions with identical numbers of ghosts. The effect of glyceraldehyde-3-phosphate dehydrogenase was seen only when it was present at the time of hemolysis. Addition of the enzyme immediately after hemolysis but before restoration of isotonicity, or during the healing period did not produce leaky ghosts. If hemolysis occurred in the presence of enzyme which had been inactivated by iodoacetic acid, resealing was also inhibited.

Since it was not possible to measure 3-*O*-methylglucose entry in ghosts which remained permeable to L-glucose, the effect of glyceraldehyde-3-phosphate dehydrogenase on transport was tested at a concentration of 0.25 mg/ml where resealing could take place. Ghosts prepared with this concentration of enzyme had a V_s of 40.9 ± 5.5 pmol/10^9 ghosts · min^{-1} ($n = 4$) and an F_s of 5.87 ± 1.30 μl/10^9 ghosts · min ($n = 4$).

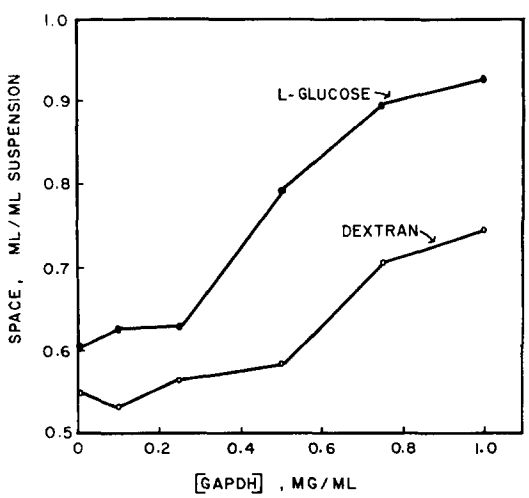

Fig. 3. Effect of glyceraldehyde-3-phosphate dehydrogenase concentration on L-glucose and dextran spaces of ghost suspensions. Packed red cells were reversibly hemolyzed in the presence of various concentrations of glyceraldehyde-3-phosphate dehydrogenase, and the ghosts healed for 60 min, washed, and the L-glucose (●) and dextran (○) spaces measured as described in Experimental Procedures. Total water of the suspensions was 0.9566 ± 0.0005 ml/ml suspension. The entire concentration curve was run three times and portions of it 4–5 times, with the same results.

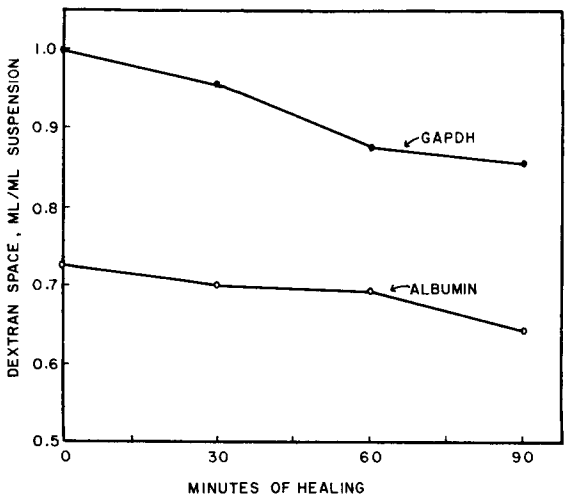

Fig. 4. Effect of the time allowed for resealing of ghosts prepared in the presence of glyceraldehyde-3-phosphate dehydrogenase or albumin. Cells were reversibly hemolyzed in the presence of 1 mg/ml glyceraldehyde-3-phosphate dehydrogenase (●) or albumin (○), healed for various times at 37 °C, and the dextran spaces determined as described in Experimental Procedures. The number of ghosts/ml suspension was $1.73 \cdot 10^9$ and $1.76 \cdot 10^9$ for glyceraldehyde-3-phosphate dehydrogenase and albumin treated ghosts respectively.

Phloretin reduced the V_s and F_s to 5.96 ± 2.59 pmol/10^9 ghosts · min^{-1} and 1.58 ± 0.53 μl/10^9 ghosts · min ($n = 3$) respectively.

Ghosts were also prepared by reversibly hemolyzing cells in the presence of glyceraldehyde-3-phosphate dehydrogenase and restoring the osmolarity to 125 mosM/l and pH 6.0. These conditions were used to promote enzyme binding to the membrane [7]. In the lower ionic strength medium, ghost membranes again did not reseal and therefore transport of 3-*O*-methylglucose could not be assessed. In addition, the lower pH and osmolarity appeared to stimulate transport in intact cells (unpublished observations). On the basis of these observations, glyceraldehyde-3-phosphate dehydrogenase specifically affects membrane resealing but may not be a regulator of transport.

DISCUSSION

Reversible hemolysis of avian erythrocytes has previously been used as a technique to study glycine transport [18] and 3-*O*-methylglucose efflux [3]. In these experiments the ghosts were found to demonstrate active and facilitated transport. In the present experiments, a similar hemolyzing fluid was used, but the ratio of medium to cells, temperature, pH and healing time were altered to provide a more homogeneous ghost population. The loss of cellular material was greater in the present experiments since the ghosts were pale pink in color rather than "cherry red" [18], and had lost approximately 80 % of their dry weight. The concentration of calcium in the restored hemolysate, although higher than that used by Lepke and Passow [13] had no effect on the rate of 3-*O*-methylglucose entry in ghosts, or on ghost volume or proportion of ghosts regaining impermeability to sugar. Monovalent cation permeability was not

tested in these ghosts, since extracellular concentrations of these ions were previously found to be without effect on transport in intact cells.

In order to characterize carrier-mediated transport in reversibly hemolyzed cells, it was necessary to define the extracellular space, which in these entry experiments would include the space within ghosts which were permeable to sugar. The usual centrifugation methods for determination of hematocrit and extracellular volume could not be used for ghost suspensions because they did not pack well in the microhematocrit tubes. Dextran and inulin have been widely used as extracellular markers for intact cells. The assumption that dextran could also be used as an extracellular marker in ghost suspensions received support from the fact that the dextran and inulin spaces were the same in resealed ghosts in spite of the large difference in size of the markers. In addition, the mean cell volume as determined by the Coulter Counter was in good agreement with the mean cell volume calculated from the dextran space and the number of particles in the suspension. L-glucose spaces in ghosts prepared in the absence of protein, or with albumin, lactic dehydrogenase or peroxidase, were the same as the dextran spaces, indicating that nearly all of the ghosts had regained their permeability barrier to L-glucose. Such ghosts could logically be used to study sugar entry rates.

Ghosts exhibited a different response to current than intact cells, as seen by the more rapid changes in apparent mean cell volume when the aperture current was increased in the Coulter Counter. Cell membranes [19] and bacterial membranes [20] both exhibit dielectric breakdown and increased permeability with increased electric field strengths or increased detector current in the Coulter Counter. The sharp decrease in apparent mean cell volume of ghosts between 0.5 and 2.0 mA in these experiments could indicate that ghost membranes were broken down more readily. This may suggest that the membrane organization in ghosts is not quite the same as in the intact cells, or that the ghost membrane becomes leaky more easily after the loss of intracellular compounds which were bound to the membrane and exerted inhibitory effects on permeability.

Carrier-mediated 3-*O*-methylglucose entry in ghosts was significantly faster than that in intact cells, as seen by the 10-fold increase in V_s and F_s, and by the ability of phloretin to inhibit the entry. The greater effect of phloretin on sugar entry in ghosts than in intact cells demonstrated that the entry of sugar by carrier mediated transport was a greater proportion of the total sugar entry than it was in intact cells. Increased rates of sugar entry are consistent with the previous report of stimulation of 3-*O*-methylglucose exit after hemolysis, without an increase in the rate of sorbitol exit [3]. The increased transport rate was not due to shrinkage of the cells, since the ghosts were larger than intact cells and since the method of calculating V_s and F_s included terms for the intra- and extracellular water and ghost cell number.

Two possible explanations for increased transport rates are (1) membrane reorganization, to the extent that the carrier can change conformation more readily and (2) loss of a molecule(s), which in the intact cell, inhibited carrier movement. Many studies with human erythrocyte ghosts suggest that permeability barriers to ions, small molecules and proteins, and activities of membrane-related enzymes can either be retained or increased, depending upon methods of ghost preparation [21]. In view of these data and the behavior of ghost membranes in the Coulter Counter, membrane reorganization could be a possible mechanism of transport stimulation by

reversible hemolysis, but it cannot as yet be distinguished from a mechanism involving regulation by compounds bound to the membrane.

Loss of an intracellular regulator of transport is a possibility which has not been proved or disproved. Although glyceraldehyde-3-phosphate dehydrogenase clearly changed the membrane properties enough to prevent resealing of ghosts, its effect, if any, on transport could not be thoroughly tested. At a concentration which did allow resealing, the enzyme was added to the hemolyzing fluid in the absence or the many factors which are known to affect its activity [22] and binding to membranes [7, 8, 23–25]. A complete screening of these factors has not been done.

The technique of reversible hemolysis of avian erythrocytes appears to be a good method of studying transport of non-electrolytes as long as the ghost population can be defined in terms of its competent or tightly resealed ghosts. It has an advantage over vesicle preparations since the volume of intracellular water is large enough to allow entry to be easily studied, and it allows testing of potential transport regulators. However, the limitation of the total osmolarity at the time of hemolysis restricts testing of combinations of compounds at higher concentrations. The avian erythrocyte is a valuable model to study since these cells have a regulated rate of sugar transport [3, 4] which is altered by reversible hemolysis, thus presenting the possibility of defining the regulatory mechanisms which were lost.

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